

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 200512.00002

In re International patent application of

CASE WESTERN RESERVE UNIVERSITY

International Application No. PCT/IB05/050257

International Filing Date: January 21, 2005

For: HYBRID AND CHIMERIC POLYPEPTIDES THAT REGULATE ACTIVATION OF
COMPLEMENT

Commissioner for Patents
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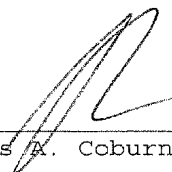
STATEMENT ACCOMPANYING SEQUENCE LISTING

Dear Sir:

The undersigned hereby states that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing.

Respectfully submitted,

Nov. 3, 2005
Date


James A. Coburn

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MCP works by binding to the C3b and C4b present on the cell surface thereby targeting C3b and C4b for degradation by factor I, a plasma protease, and thereby destroying any subsequent C3 or C4 convertase activity. Thus, MCP is said to have "cofactor activity". Because MCP is localized on the cell surface, it protects only the cells on which it is present and is therefore said to act in an intrinsic manner. The sequence of a cDNA encoding human MCP has been reported by Lublin et al, J. Exp. Med., (1988) 168:181-194. The nucleotide sequence of a cDNA encoding MCP is shown in Fig 4B (SEQ ID NO: 6).

Table 2

Amino Acid	Domain
1-34	Leader peptide
35-95	CCP
96-158	CCP
159-224	CCP
225-285	CCP
286-314	STP
	B-domain: VSTSSTTKPASSAS (SEQ ID NO: 26)
	C-domain: GPRPTYKPPVSNP (SEQ NO: 27)
315-327	Undefined segment
328-351	Transmembrane domain
352-361	Intracytoplasmic anchor
362-377	Cytoplasmic tail one: TYLTDETHREVKFTSL (SEQ ID NO: 28)
362-384	Cytoplasmic tail two: KADGGAEYATYQTKSTTPAEQRC (SEQ ID NO: 29)

[0015] Effects of Excessive Activation of Complement

[0016] Excessive activation of complement causes damage to normal host tissues in a number of conditions. Some diseases in which complement is known to be activated include systemic lupus erythematosus, acute myocardial infarction, burn, sepsis, stroke and the adult respiratory distress syndrome. Accordingly, it is desirable to have soluble agents that can block complement activation. Such agents would be useful for treating the above-mentioned human diseases and a wide range of other diseases (See Table 3 below). The construction of hybrid complement regulatory proteins has been attempted previously, but with mixed results. For example, a hybrid containing CCPs 1-4 of MCP and CCPs 1-4 of DAF was

[0017] Fig. 1A is a listing of the amino acid sequence of human Decay Accelerating Factor (DAF), SEQ. ID NO: 1;

[0018] Fig. 1B is a listing of a DNA sequence encoding DAF, SEQ. ID NO: 2;

[0019] Fig. 2 is a listing of the amino acid sequence of human Complement Receptor 1 (CR1), SEQ. ID NO: 3;

[0020] Fig. 3 is a listing of a DNA sequence encoding CR1, SEQ. ID NO: 4;

[0021] Fig. 4A is listing of the amino acid sequence of human Membrane Cofactor Protein (MCP), SEQ. ID NO: 5;

[0022] Fig. 4B is a listing of a DNA sequence encoding MCP, SEQ. ID NO: 6;

[0023] Fig. 5 is a representation of a lipid tail structure (SEQ ID NO: 25);

[0024] Fig. 6A and 6B are listings of PCR primers DSIGEB and DAF3P, SEQ. ID NO's: 7 and 8, respectively;

[0025] Figs. 7A- 7D are listings of PCR primers CR1094X, CR1099N, CR135ON, and CR1B3P, SEQ. ID NO's: 9-12, respectively;

[0026] Fig. 8A is a listing of the amino acid sequence of the protein DAF-CR1B, SEQ. ID NO: 13;

[0027] Fig. 8B is a listing of a DNA sequence encoding DAF-CR1B, SEQ. ID NO: 14;

[0028] Fig. 9A is a listing of the amino acid sequence of protein DAF – CR1BB, SEQ. ID NO: 15;

[0029] Fig. 9B is a listing of a DNA sequence encoding DAF – CR1BB, SEQ. ID NO: 16;

[0030] Fig. 10A and 10B are listings of the PCR primers IgG45 and IgG43, SEQ. ID NO's: 17 and 18, respectively;

[0031] Fig. 11A is a listing of amino acid sequence of protein DAF – IgG4, SEQ. ID NO: 19;

[0032] Fig. 11B is a listing of a DNA sequence encoding DAF – IgG4, SEQ. ID NO: 20;

[0033] Fig. 12A and 12B are listings of the PCR primers MCP5 and MCP3, SEQ. ID NO's: 21 and 22, respectively;

[0034] Fig. 13A is a listing of the amino acid sequence of protein DAF – MCP, SEQ ID NO: 23;

[0035] Fig. 13B is a listing of a DNA sequence encoding DAF – MCP, SEQ. ID NO: 24;

[0036] Fig. 14 is a Western blot of protein samples containing hybrid proteins probed with monoclonal antibodies raised against DAF and CR1;

[0037] Fig. 15 is a Western blot of a protein samples containing DAF – MCP probed with monoclonal antibodies raised against DAF and MCP;

[0038] Fig. 16 is a graph showing the percent inhibition of hemolysis of DAF – CR1BB and sCR1 in a whole serum assay;

[0039] Fig. 17 is a graph showing the percent inhibition of hemolysis of DAF – MCP and DAF in a whole serum assay;

[0040] Figs. 18A and 18B are graphs showing the percent inhibition of hemolysis of the hybrid proteins in a classical pathway C3 convertase assay;

[0041] Fig. 19 is a graph showing the percent inhibition of hemolysis of DAF – CR1B and DAF in a classical pathway C5 convertase assay;

[0042] Fig. 20 is a graph showing the percent inhibition of hemolysis of DAF – CR1BB, sCR1 and DAF-CR1B in a classical pathway C5 convertase assay;

[0043] Fig. 21 is a Western blot of supernatants of cells expressing the hybrid proteins of DAF – MCP or DAF-CR1BB with and without factor I in a cofactor assay.

the hybrid protein DAF - CR1B was constructed using *DAF13.2.U/pBTKS* and two primers *DSIGEB* and *DAF3P* in a PCR reaction (Vent polymerase [New England Biolabs] with the following times: 94°C 3min [initial melting]; 94°C 1min, 55°C 1min15sec, 72°C, 1min15sec for 25 cycles; and 72°C 7min [final extension]). *DSIGEB* is a 42 nucleotide ("nt") primer that has the sequence 5'-ATA TAC GAA TTC AGA TCT ATG ACC GTC GCG CGG CCG AGC GTG-3' (Fig. 6A SEQ. ID NO:7). *DAF3P* is a 35nt primer that has the sequence 5'-ACA GTG CTC GAG CAT TCA GGT GGT GGG CCA CTC CA-3' (Fig. 6B, SEQ. ID NO:8). The resultant PCR product was named *DAF1*. It contained DAF's signal sequence followed by CCPs 1, 2, 3 and 4 ending with cysteine 249 (Cys-249) in CCP4. Upstream of the signal sequence, two restriction enzyme sites were built in, BglIII (A▼GATCT) and 5' of BglIII, EcoRI (G▼AATTC). Three prime (3') of CCP4 and encompassing part of the Cys-249 codon (TGC), the restriction enzyme site XhoI (C▼TCGAG) was inserted. *DAF1* was subcloned into *pT7B* and fully sequenced.

[00108] The CR1 portion of the hybrid protein DAF - CR1B was constructed using *CR1/AprM8*. *CR1/AprM8* was cut with the restriction enzyme NsiI (ATGCA▼T) releasing several pieces, two of which were recovered (1094nts and 1350nts) and subcloned into *pGEM7Zf(+)*. The "1094" fragment (encompassing nts 557 to 1670 of CR1) was amplified by PCR using the primers *CR1094X*(5') and *CR1094N*(3'). *CR1094X* is a 41nt primer having the sequence 5'-ATA TAC CTC GAG TCC TAA CAA ATG CAC GCC TCC AAA TGT GG-3' (Fig. 7A, SEQ ID NO:9). It has an XhoI site. *CR1094N* is a 34nt primer having the sequence 5'-ACA GTG ATG CAT TGG TTT GGG TTT TCA ACT TGG C-3' (Fig 7B, SEQ ID NO:10). It has an NsiI site. This set of primers produces a sequence from the linker between CCP3 and CCP4 of CR1 into CCP8 of CR1. PCR conditions were the same as those for *DAF1*. The "1350" fragment, encompassing nts 1671 to 3020 of CR1, was amplified by PCR using primers *CR1350N*(5') and *CR1B3P*(3'). *CR1350N* is a 41nt primer having the sequence 5'-ATA TAC ATG CAT CTG ACT TTC CCA TTG GGA CAT CTT TAA AG-3' (Fig. 7C, SEQ ID NO: 11). It has an NsiI site. *CR1B3P* is a 57nt primer having the sequence 5'-ACA GTG AGA TCT TTA GTG ATG GTG ATG GTG ATG AAT TCC ACA GCG AGG GGC AGG GCT-3' (Fig. 7D, SEQ ID NO: 12). It has a BglIII site. PCR conditions were the same as those for *DAF1* except the 25 cycle extension time at 72° was 2min, not 1min15sec. This set of primers produces a sequence from CCP8 of CR1 to the end of CCP14 (in LHRB, specifically, ...SSPAPRCGI (SEQ ID NO: 30)) with a C-terminal 6XHis tag (SEQ ID NO: 31) and stop codon. These PCR fragments were subcloned into *pT7B*.

[00109] It is noteworthy that the natural linker between CCP3 and CCP4 of CR1 is the amino acid sequence "IIPNK" (SEQ ID NO: 32) (see Fig. 2, SEQ. ID NO: 3). Due to the insertion of the XhoI restriction site, the hybrid protein's linker between DAF CCP4 and CR1 CCP4 is "SSPNK" (SEQ ID NO: 33) (see Fig. 8A (SEQ. ID NO: 13).

[00110] DNA sequence the data obtained confirmed the presence of the correct nucleotide sequences.

[00111] The vector pSG5 (Stratagene) was cut with the restriction enzymes EcoRI and BglII to accommodate the insertion of *DAF1* (EcoRI to XhoI), *CR828XN3*(XhoI to NsiI) and *CR1300NBF* (NsiI to BglII). The vector and the three fragments were ligated using Promega T4 DNA ligase, and transformed into DH5 α maximum efficiency competent cells. Agarose gel electrophoresis confirmed the presence of the vector and insert. The cDNA from one colony was used for transfection into COS cells using Lipofectamine (Invitrogen) reagent. The supernatant was harvested two days later. Western blots using 2H6, an anti-DAF CCP4 antibody, and an anti-His tag antibody indicated the presence of the hybrid protein. The amino acid sequences of the DAF – CR1B is provided in Fig. 8A (SEQ. ID NO: 13). A DNA sequence encoding DAF –CR1B is provided in Fig. 8B (SEQ. ID NO: 14).

[00112] EXAMPLE 2: Hybrid Protein DAF - CR1BB

[00113] Another hybrid protein, DAF - CR1BB, was prepared by recombinant techniques. DAF - CR1BB, comprises DAF's four CCPs, a spacer comprised of CCPs 4-7 of CR1, separating the functional unit of DAF from a first cofactor 1 functional unit of CR1, LHR B, and a second spacer, CCPs 11-14 of CR1, separating the first cofactor 1 functional unit of CR1 from the second cofactor 1 functional unit of CR1, also LHR B. More specifically, DAF - CR1BB was prepared by adding an additional LHRB of CR1 to DAF - CR1B To add the additional cofactor LHR, DAF - CR1B was cut with BamHI and a BamHI fragment (nucleotide #1861 to 3210) from CR1 in AprM8 was introduced. The BamHI fragment could enter the plasmid in either the correct or reverse orientation. Screening with SmaI found several clones with the correct nucleotide orientation. The amino acid sequence of DAF - CR1BB is provided in Fig 9A (SEQ ID NO: 15). The DNA sequence encoding DAF - CR1BB is provided in Fig 9B (SEQ ID NO: 16)

sequence. DH5 α max competent cells were transformed with the ligation mix. Plasmid DNA from resulting colonies were screened by cutting the DNA with BglIII and examining the resulting band by agarose gel electrophoresis. Plasmid DNA was purified and cDNA was checked (uncut and BglIII-cut). The amino acid sequence of DAF - IgG4 is provided in Fig 11A (SEQ. ID NO: 19). The DNA sequence encoding DAF - IgG4 is provided in FIG 11B (SEQ. ID NO: 20)The cDNA was transfected into COS cells.

[00118] Note that the IgG45 primer codes for a slightly different link between CR1 CCP7 and the Hinge of IgG4 (in DAF - IgG4) than the link between CR1 CCP7 and MCP CCP1 (in "DAF - MCP" see Example 4). IgG45 results in "GILV" (SEQ ID NO: 34) ("V" is the last amino acid of the CH1 domain of IgG4) instead of "GILGH" (SEQ ID NO: 35) which is found in DAF - MCP and is also the normal link between CR1 CCP7 and CCP8 (and is therefore what is found in DAF - CR1B and DAF - CR1BB hybrids).

[00119] EXAMPLE 4 Hybrid Protein DAF - MCP

[00120] A hybrid protein, referred to hereafter as DAF -MCP, comprising a decay accelerating functional unit of DAF, a cofactor 1 functional unit derived from MCP, and a spacer derived from CR1 was prepared (DAF CCPs 1,2,3,4- CR1 CCPs 4,5,6,7- MCP CCPs 1,2,3,4+ 2 amino acids (VS) of MCP STP region + 6xHis (SEQ ID NO: 31)). MCP cDNA (with 3'-end sequence encoding GPI-anchor addition) in PEE14 was used. More MCP cDNA in DH5 α (Wizard SV DAF - IgG4-prep) ("MCP-GPI (A)") was subsequently prepared. Primers for the MCP portion of DAF -MCP are:

MCP5: 5'-ATA TAC GAA TTC TGG GTC ACT GTG AGG AGC CAC CAA CAT TTG AAG C-3' (Fig. 12A, SEQ ID NO: 21); and

MCP3: 5'-ACA GTG AGA TCT TTA GTG ATG GTG ATG GTG ATG CGA CAC TTT AAG ACA CTT TGG AAC-3' (Fig. 12B, SEQ. ID NO: 22).

[00121] The PCR reaction used Vent polymerase from New England Biolabs. The MCP PCR fragment was cut with EcoRI ("E") and BglIII ("B") in Promega Buffer H ("H"). The Quick Ligase method (New England Biolabs) was used to ligate E/B/H-cut MCP PCR and E/B/H-cut pSG5. DH5 α maximum efficiency competent cells were transformed with the mixture. Colonies were picked, the DNA was extracted from the bacteria and cut with

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sequence. DH5amax competent cells were transformed with the ligation mix. Plasmid DNA from resulting colonies were screened by cutting the DNA with BglII and examining the resulting band by agarose gel electrophoresis. Plasmid DNA was purified and cDNA was checked (uncut and BglII-cut). The amino acid sequence of DAF - IgG4 is provided in Fig 11A (SEQ. ID NO: 19). The DNA sequence encoding DAF - IgG4 is provided in FIG 11B (SEQ. ID NO: 20) The cDNA was transfected into COS cells.

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[00120] A hybrid protein, referred to hereafter as DAF -MCP, comprising a decay accelerating functional unit of DAF, a cofactor 1 functional unit derived from MCP, and a spacer derived from CR1 was prepared (DAF CCPs 1,2,3,4- CR1 CCPs 4,5,6,7- MCP CCPs 1,2,3,4+ 2 amino acids (VS) of MCP STP region + 6xHis (SEQ ID NO: 31)). MCP cDNA (with 3'-end sequence encoding GPI-anchor addition) in PEE14 was used. More MCP cDNA in DH5α (Wizard SV DAF - IgG4-prep) ("MCP-GPI (A)") was subsequently prepared. Primers for the MCP portion of DAF -MCP are:

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MCP3: 5'-ACA GTG AGA TCT TTA GTG ATG GTG ATG GTG ATG CGA CAC TTT AAG ACA CTT TGG AAC-3' (Fig. 12B, SEQ. ID NO: 22).

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